

SPECIFIC ANTIBODY WITHIN LYMPHOID GERMINAL CENTER
CELLS OF MICE AFTER PRIMARY IMMUNIZATION WITH
HORSERADISH PEROXIDASE: A LIGHT AND ELECTRON
MICROSCOPIC STUDY*

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Our present knowledge of the development, function, and fate of germinal centers of lymphatic tissue and, in particular, of lymphoid germinal center cells (germinoblasts, germinocytes) is still incomplete. Evidence obtained from phylogenetic (1), ontogenetic (2), and kinetic (3) studies points to an intimate association of germinal centers with the capacity to produce large amounts of antibody, especially in relation to anamnestic responses (see review, 4). The presence of immunoglobulins within germinal centers was first demonstrated by Leduc et al. (5) and White et al. (6) with the aid of the fluorescent antibody technique in popliteal lymph nodes of rabbits immunized with diphtheria toxoid and ovalbumin. In these early studies specific antibody was reported to be distributed either in a reticular pattern between follicle cells or, although less frequently and less distinctly, within germinal center cells. A similar distribution of immunoglobulins within human lymphoid tissue was described by Ortega and Mellors (7). White (8) noted that the presence of antibody-containing germinal centers 4 days after secondary antigenic stimulation with ovalbumin and diphtheria toxoid was limited to the lymph node regional to the site of primary antigen injection given 3 wk before. In later studies White et al. (9, 10) reported antibody-containing cells within Malpighian bodies of chicken spleen from between days 14 to 28 after a single intravenous injection of human serum albumin.

Recently, Avrameas et al. (11) and Straus (12) have introduced the use of antigenic and catalytic properties of plant and heterologous animal enzymes for detecting anti-enzyme antibody at the cellular level. Horseradish peroxidase (HRP) appears to be particularly well suited for evaluation by light and electron microscopy since 98-100% of the enzymatic activity of HRP was found to be

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retained after its combination with specific antibody¹. The localization of a positive reaction can easily be recognized by light microscopy, and it is possible to use conventional counterstaining procedures. Moreover, the formation of an electron-dense reaction product after interaction of the enzyme with a suitable substrate and acceptor system provides for reliable ultrastructural analysis.

The origin of immunoglobulins or specific antibody in germinal centers is still controversial since both the synthesis of antibody within germinal center cells and entry of circulating antibody need to be considered. The situation in which immunoglobulins appear in an intercellular localization within germinal centers merits particular attention. The question remains to be resolved whether antibody is produced by, or whether it is merely fixed to, germinal center cells, or both. In the present communication, preliminary findings (13) on the presence of antibody-containing lymphoid germinal center cells of mice after immunization with HRP are confirmed and extended. Evidence is presented in favor of antibody formation by lymphoid germinal center cells of regional lymph nodes after primary stimulation. The persistence of antigen and/or antigen-antibody complexes between dendritic reticular cells is also demonstrated.

Materials and Methods

Animals and Immunization.—A total number of 55 female Swiss albino mice (Charles River strain) were used in this study. Experimental animals were given primary injections of aluminum phosphate-adsorbed horseradish peroxidase (A grade, Calbiochem, Los Angeles, Calif.) into both hind leg footpads. The total dose was approximately 75 μg of antigen per mouse. Groups of 2–4 mice were sacrificed in ether narcosis at each time interval, i.e., 10, 14, 17, 20, 23, 26, 35, and 40 days after primary stimulation. Control mice were injected with corresponding volumes of aluminum phosphate in saline via the same route.

Serum anti-horseradish peroxidase titers were measured by passive hemagglutination according to Avrameas et al. (14).

Preparation of Lymph Nodes for Light Microscopy.—Popliteal lymph nodes were removed and quickly frozen in isopentane which was cooled in liquid nitrogen in preparation for freeze-substitution (15–16). Substitution in anhydrous ethanol was achieved in 7 days at -80°C . Preparations were brought to room temperature, the absolute ethanol was changed, and the nodes were embedded in polyester wax (B.D.H., Poole, Dorset, England) at 40°C (17). Serial sections were cut 4 μ thick with an ordinary microtome at 12°C and spread on a solution of saturated sodium sulfate, dried overnight, dewaxed with ethanol, and hydrated. In order to detect anti-HRP antibody, slides were incubated with fluid HRP (50 $\mu\text{g}/\text{ml}$) at room temperature for 20 min and washed with phosphate-buffered isotonic saline for 5 min. HRP-activity was revealed by the incubation with hydrogen peroxide in the presence of 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, Mo.) as hydrogen donor (18). Washed sections, sometimes counterstained with gallocyanine or Giemsa solution, were dehydrated and mounted in synthetic medium. Adjacent sections not incubated with HRP but treated with H_2O_2 and 3,3'-diaminobenzidine tetrahydrochloride alone were used as controls for the presence of either antigen or spontaneous peroxidase activity.

Preparation of Lymph Nodes for Electron Microscopy.—Thin slices of popliteal lymph nodes

¹ Hess, M. W., and B. Sordat. 1968. Unpublished observation.

were prefixed with 2.5% phosphate-buffered glutaraldehyde for 30 sec-1 hr at 4°C. The slices were washed overnight in phosphate buffer at 4°C and dissected under the stereomicroscope. Selected fragments of lymph node cortex presumably containing germinal centers were subsequently treated according to the technique described by Leduc et al. (19): incubation with fluid HRP (0.5 mg/ml) under continuous stirring for 1 hr at 4°C and, after extensive washing in phosphate-buffered isotonic saline, detection of peroxidase activity by the procedure used for light microscopy (18). The fragments were then postfixed in 1% phosphate-buffered osmium tetroxide, dehydrated, and embedded in Epon 812. Sections, 1 μ thick, of individual blocks were stained with Giemsa and served as light microscopic controls. Sections for electron microscopy were selected on the basis that germinal center tissue was within the penetration zone of antigen which proved to be less than 100 μ . Fragments treated with substrate and 3,3'-diaminobenzidine tetrahydrochloride alone, i.e., without previous incubation with HRP, served as controls. Ultrathin sections without further staining, were examined in a Zeiss 9A electron microscope.

RESULTS

Light Microscopy.—Examination of lymph node sections revealed that specific antibody within germinal centers may occur in an intracellular, as well as an intercellular, localization. The intracellular type of antibody localization is characterized by a distinct cytoplasmic positivity in the majority of lymphoid cells within a given germinal center (Figs. 2 and 3). This positive reaction most often covered the entire cytoplasmic rim of these elements. In the same node, other centers gave no positive reaction at all. Thus, positive and fully negative centers were frequently found in neighboring positions. On serial sections, using other staining procedures, antibody-containing germinoblasts or germinocytes could be identified as lymphoid cells with highly basophilic cytoplasm. An intense reaction was evident in a small number of cells with more abundant cytoplasm: these elements were usually located at the borders of centers and exhibited morphological and tinctorial characteristics of immature plasma cells. Only a few scattered positive cells were observed in the surrounding perifollicular areas.

In germinal centers showing a predominantly intercellular type of antibody localization, the reaction produced an irregular, reticular, or granular pattern (Fig. 6) where the spaces between dendritic reticular elements or between the latter and lymphoid germinal center cells contain masses intensely positive for antibody. Lymphoid elements with a moderately positive cytoplasmic reaction could be discerned in some of these centers.

A combination of both intra- and intercellular positive reactions could be seen in the mixed type of antibody localization: in these centers lymphoid cells with a distinctly positive cytoplasm are surrounded by extracellular antibody. As shown in Fig. 1, this mixed pattern of antibody reactivity within germinal centers was found at time intervals after antigenic stimulation identical to those observed for localization of intracellular anti-HRP antibody.

Electron Microscopy.—Based on ultrastructural characteristics, at least two types of cells with intracellular localization of anti-HRP antibody may be

distinguished within germinal centers. On the one hand, large to medium size lymphoid cells showed a reaction indicating presence of specific antibody in the perinuclear space only. As judged from their chromatin structure, relative scarcity of cytoplasm, and absence of ergastoplasmic structures, these cells may be classified as lymphoid germinal center cells (germinoblasts and germinocytes, Fig. 4). In some instances lymphoid cells also showed a granular, dis-

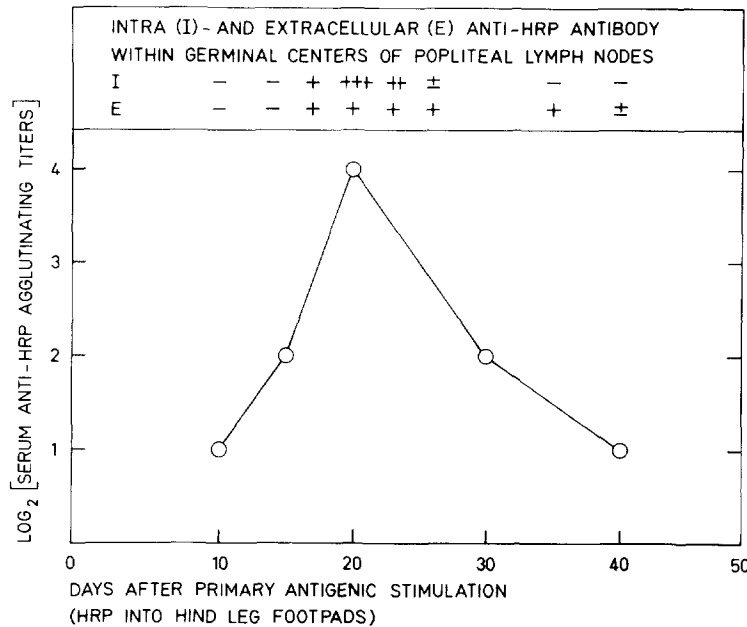


FIG. 1. Serum titers of anti-horseradish peroxidase (HRP) agglutinating antibody and appearance of anti-HRP antibody within germinal centers of regional (popliteal) lymph nodes of mice, both as a function of time after primary antigenic stimulation. The relative amounts of detectable antibody within lymphoid germinal center cells (I) and in intercellular spaces (E) were graded from small (+) to medium (++) and large (+++, see Fig. 2).

tinctly positive reaction in most of the cytoplasmic rim. On the other hand, a smaller number of cells could regularly be detected in which antibody was not only revealed in the perinuclear region but also in some structures corresponding, at least in part, to rough endoplasmic reticulum (Fig. 5). These cytoplasmic cisternae were frequently seen at one pole of the cell, near the Golgi apparatus. Occasionally, direct connections between the perinuclear space and the cytoplasmic cisternae were evident. A number of antibody-containing cells were seen in mitosis. In the centers studied so far, only questionable antibody was found within macrophages containing tingible bodies.

It should be noted that electron microscopic examination of germinal centers

composed mainly of anti-HRP-positive cells in most instances revealed the presence of extracellular antibody. Occasionally, this extracellular antibody was limited to the immediate vicinity of a positive cell, and only rarely was an exclusively intracellular positive reaction found. More often, however, extracellular antibody was associated with dendritic processes of reticular cells located between germinoblasts (Fig. 7). A few antibody-containing elements found within germinal centers, as judged by electron microscopy, were typical plasma cells.

At later time intervals after primary antigenic stimulation, anti-HRP antibody was found mainly in the spaces between reticular cells and lymphoid elements. Dendritic processes of the former were most conspicuous in the presence of larger amounts of antibody. A detailed view of irregularly distributed extracellular antibody along dendritic processes or in complex vesicular structures is given in Fig. 8.

Time Course of Appearance within Germinal Centers of Anti-HRP Antibody After Primary Stimulation.—The appearance of specific antibody in germinal centers of regional lymph nodes and in serum is presented in Fig. 1 as a function of time after antigenic stimulation. Germinal centers in which anti-HRP antibody could be demonstrated were observed at from 17 to 35 days after primary antigenic stimulation. It may be stressed that intercellular localization of anti-HRP activity appeared simultaneously with intracellular positivity, but the former remained detectable for a longer period of time. At 20 days after stimulation when serum antibody titer was highest, anti-HRP-positive centers were more numerous and more prominent than before or after that time interval. At day 40 after injection of antigen, it was not possible to demonstrate germinal center-associated anti-HRP activity. It should be emphasized that at all time intervals after antigenic stimulation, germinal centers could be found which remained negative when tested for the presence of anti-HRP antibody.

Controls and Presence of Antigen in Germinal Centers.—Cells and tissues of nonimmunized animals, except for erythrocytes, neutrophilic and eosinophilic granulocytes, some histiocytes, and questionably mast cells, never showed spontaneous peroxidase activity under the conditions of the experiment and with the techniques used. Since these cell types can easily be identified by both light and electron microscopy and were not observed within germinal centers in the present study, endogenous peroxidase activity did not interfere with the detection of anti-HRP antibody. Lymph nodes of immunized animals were also examined without previous incubation *in vitro* with HRP. In these, enzymatically active antigen was observed by electron microscopy in intercellular spaces between dendritic reticular cells and lymphoid elements of germinal centers as long as 35 days after primary injection of HRP (Fig. 9). Peroxidase activity at this site was never observed in nonimmunized controls.

DISCUSSION

Studies on *de novo* formation and development of germinal centers are facilitated by injecting the antigen into areas drained by so-called oligosynthetic lymph nodes such as the popliteal which contain no or only few of these structures in nonimmunized animals (3). Several questions concerning the present methods need to be discussed before an interpretation of the above findings can be given. According to Leduc et al. (19), penetration of HRP (mol wt 40,000) within tissue fragments prefixed for electron microscopy is restricted and, within germinal centers, appeared to be limited to less than 100 μ . As a consequence of restricted diffusion of antigen, anti-HRP antibody could not be identified in germinal centers or germinal center cells located beyond the penetration zone of HRP. Antigen entry into single cells may also be affected by prefixation of tissue. A striking difference was observed between light- and electron microscopic findings. With light microscopic examination, anti-HRP antibody was found to be evenly distributed throughout the cytoplasm of positive cells, whereas in electron microscopic preparations, antibody most often was limited to the perinuclear space and, occasionally, to a few primitive ergastoplasmic structures. Since the cut surfaces of tissue sections prepared for light microscopy and obtained after freeze-substitution were exposed to the antigen solution, contact of HRP with intracytoplasmic specific antibody was possible without impediment by structures such as cellular or intracellular membranes. In addition, prefixation with aldehydes, particularly with glutaraldehyde, may cause denaturation of immunoglobulins (20). The influence of prefixation upon detection of anti-HRP activity in the cytoplasm, particularly near ribosomes, has recently been discussed for other experimental systems by Leduc et al. (21). It should be emphasized, however, that the conditions considered above influence the interpretation of the experimental results only insofar as factors that may prevent access of antigen to the corresponding antibody could give negative results; under the conditions used, no interference with false positive reactions is to be expected.

Specific anti-HRP antibody could first be demonstrated within lymphoid cells of newly formed germinal centers, together with extracellular antibody, at approximately 2 wk after antigenic stimulation. This finding corresponds to the results of previous kinetic studies indicating delayed formation of germinal centers after true primary antigenic stimulation (22). At later time intervals after primary injection of antigen, intercellular anti-HRP activity appeared to persist, whereas the number of detectably antibody-containing cells declined. Theoretically, anti-HRP antibody found within a lymphoid germinal center cell could have been either produced by the cell itself or taken up from the outside. The latter possibility can be regarded as highly unlikely since (a) some germinoblasts containing antibody in the perinuclear space could be observed without antibody being present in adjacent tissue, (b) perinuclear

localization of anti-HRP antibody has been reported to be characteristic of immature plasma cells in early stages of active antibody production (19), and (c) intercellular localization of anti-HRP antibody was not observed prior to the appearance of an intracellular positive reaction in lymphoid germinal center cells. In view of an earlier report by Pernis et al. (23), it seems probable that some of the intercellular antibody originated from the circulating blood and became fixed within germinal centers. This possibility should be considered particularly with regard to later phases of the primary response.

Since germinal centers are sites of intense mitotic activity, the present findings provide strong support to the view that germinoblasts are immature antibody-producing elements which pass through a phase of combined proliferation and differentiation. The ultimate fate of germinal center cells still needs to be clarified. Kinetic (24) and immunohistochemical studies (25) on germinal centers of the tonsil, an organ exposed to constant antigenic stimulation, revealed a well-defined and unidirectional pathway of migration and differentiation of lymphoid cells which finally emigrate towards the surface. The migrational behavior of germinal center cells in lymph nodes remains to be studied in more detail.

The observation that both antibody-containing cells and extracellular antibody were present in some germinal centers while other centers remained negative throughout the observation period, may be tentatively interpreted as an expression of monospecificity of these structures with regard to the antigen stimulating their development. A similar suggestion was made by Hanna et al. (26), based on antigen-trapping in germinal centers. Antigen-trapping, a phenomenon originally described by Nossal et al. (27) and subsequently studied by McDevitt et al. (28) may well be initiated and/or enhanced through corresponding antibody produced locally by lymphoid germinal center cells. The absence of antigen-trapping after a single injection of antigen in specifically tolerant (29-30), in germfree (31), and in irradiated animals (32-33) could thus be interpreted on the basis of absence or repression of antibody synthesis in lymphoid germinal center cells.

Intercellular fixation within germinal centers of circulating antibody could, at least in part, be interpreted as a secondary phenomenon: locally produced antibody with low avidity (34) may combine with antigen; the resulting complexes contain free antigenic determinants with which, in a later phase, extraneous antibody may react. Since, with increasing amounts of extracellular antibody the dendritic processes of germinal center reticular cells became more prominent and numerous, it is possible that these processes develop as a consequence of the presence of antigen-antibody complexes, and are not preexistent, at least not to the same extent. Prolonged persistence of antigen in the intercellular spaces of germinal centers, as shown in the present study, possibly also depends on the presence of specific antibody not always detectable by immunohistochemical techniques.

SUMMARY

The appearance in mice of specific antibody within newly formed germinal centers in lymph nodes was demonstrated by light and electron microscopy after regional primary antigenic stimulation with horseradish peroxidase (HRP).

Lymphoid germinal center cells containing anti-HRP antibody in the perinuclear space and in the cytoplasm were detected from 17 to 26 days after antigenic stimulation. Extracellular anti-HRP antibody within germinal centers, localized between dendritic reticular cells and lymphoid elements, could not be found before the appearance of intracellular antibody. These findings strongly suggest antibody formation by lymphoid germinal center cells. Both antigen and corresponding antibody persisted in intercellular spaces up to 35 days after primary stimulation.

The concomitant presence in a given lymph node of germinal centers which are positive or negative with regard to specific antibody provide evidence in favor of monospecificity of individual centers. The mechanisms of antigen-trapping within germinal centers are discussed in the light of the present observations.

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Explanation of plates: The photomicrographs shown in Figs. 2-8 illustrate the presence of anti-horseradish peroxidase (HRP) antibody in germinal centers of mouse popliteal lymph nodes at various time intervals after primary stimulation with HRP via the hind leg footpads. Sections of fixed tissue were prepared for light microscopy (Figs. 2, 3, and 6) using the freeze substitution technique and were incubated with HRP to permit binding to its corresponding antibody. Peroxidase activity was then revealed by incubation with hydrogen peroxide in the presence of 3,3'-diaminobenzidine. Sections for electron microscopy (Figs. 4, 5, 7, and 8) in which germinal center tissue was within the penetration zone of HRP were prepared according to the method described by Leduc et al. (19). Combined histochemical and electron microscopic demonstration of antigen (Fig. 9) was possible in germinal center tissue which had not been exposed to, or penetrated by, HRP in vitro.

FIG. 2. Germinal center containing anti-HRP antibody, 20 days after primary stimulation. This reaction was graded as +++ . × 200.

FIG. 3. Germinal center at higher magnification, illustrating the predominantly intracellular localization of anti-HRP antibody, 20 days after primary stimulation. Note that a large number of lymphoid center cells exhibit a positive reaction over the entire cytoplasmic rim. × 700.

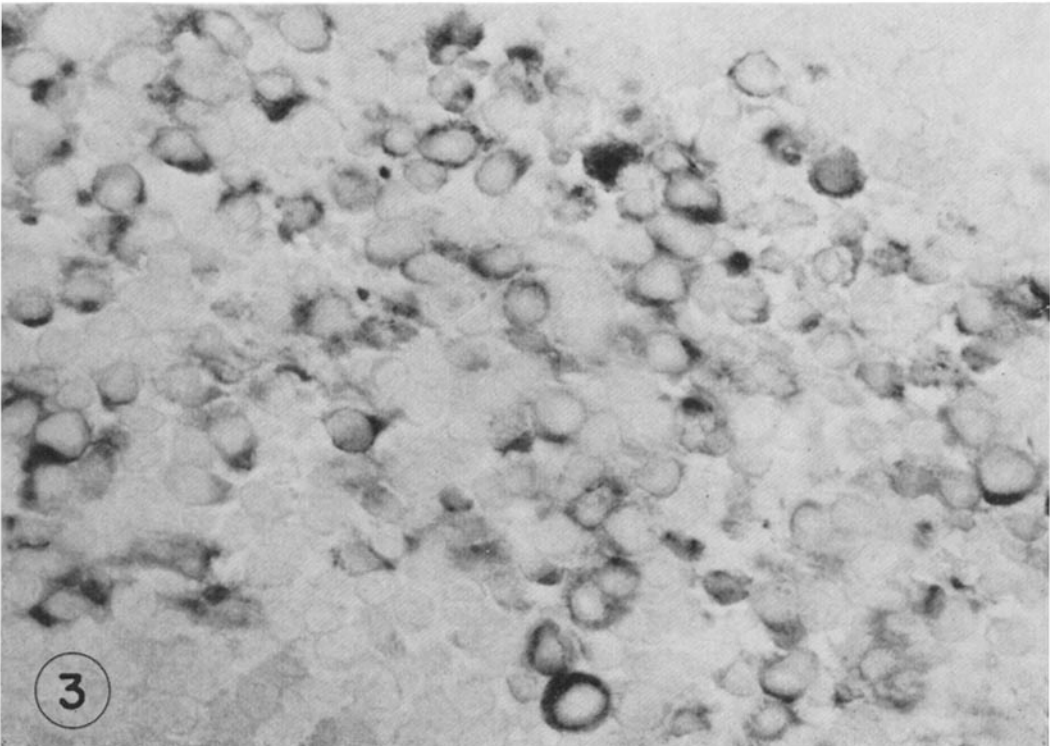
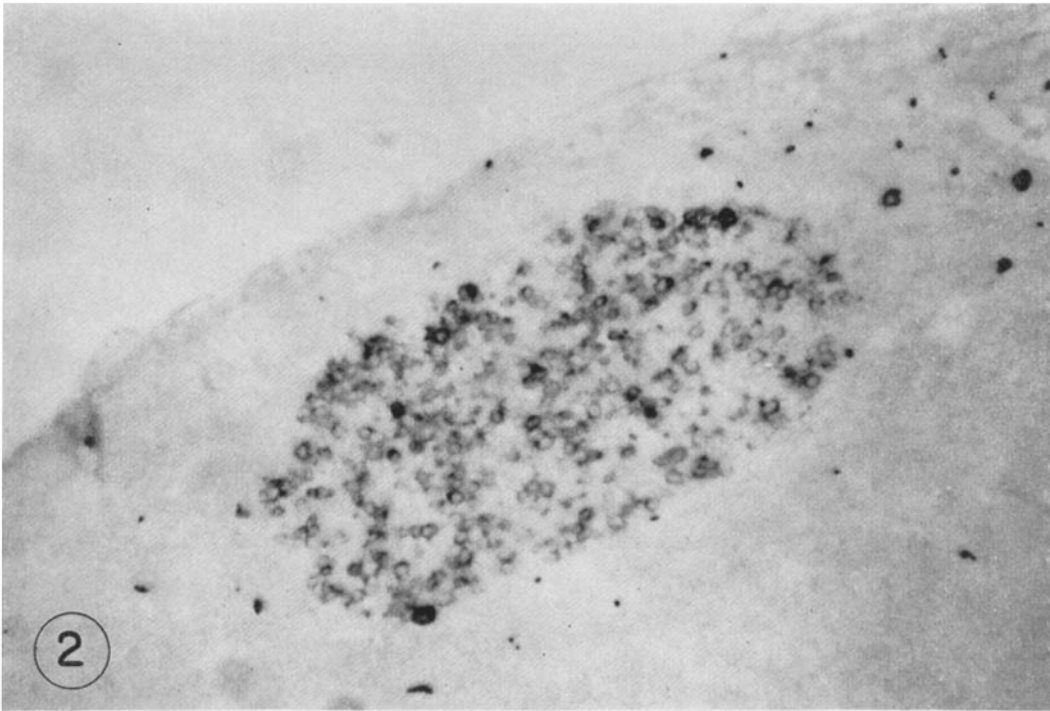
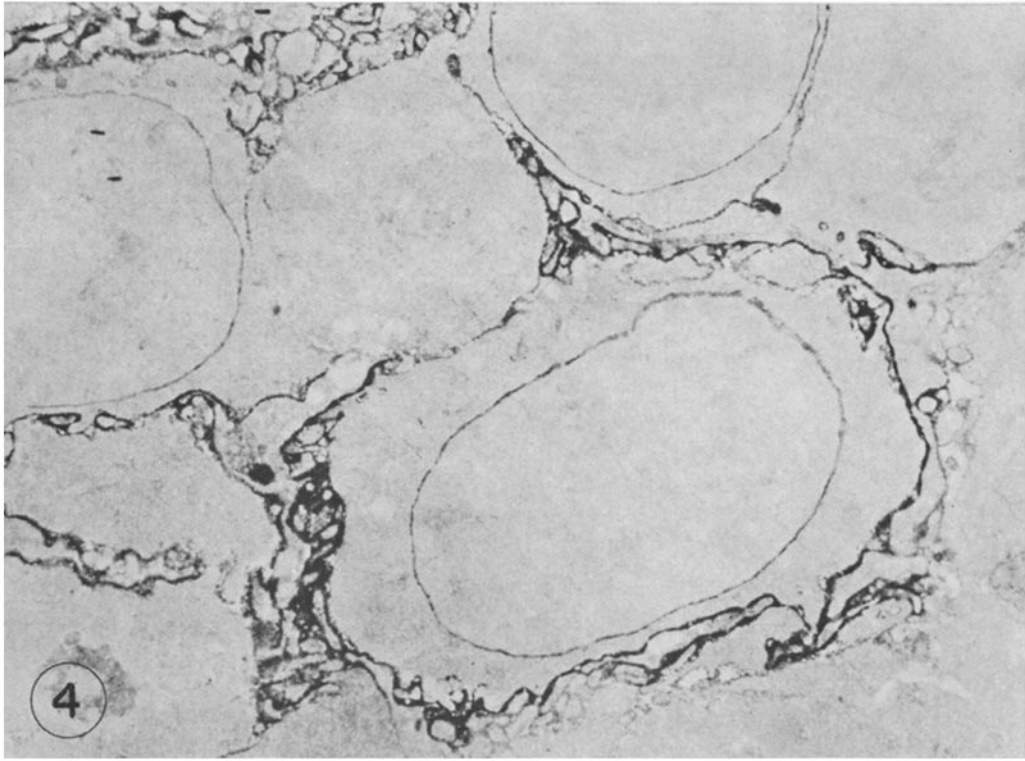


FIG. 4. Combined immunohistochemical and electron microscopic demonstration of anti-HRP antibody in the perinuclear space of lymphoid germinal center cells and between the former and dendritic reticular cells, 23 days after primary stimulation. $\times 8000$.

FIG. 5. Lymphoid germinal center cell containing anti-HRP antibody in the perinuclear space and in primitive ergastoplasmic cisternae. In other lymphoid elements (left) the positive reaction is confined to the perinuclear space. This lymph node was also obtained 23 days after primary stimulation. $\times 11,000$.



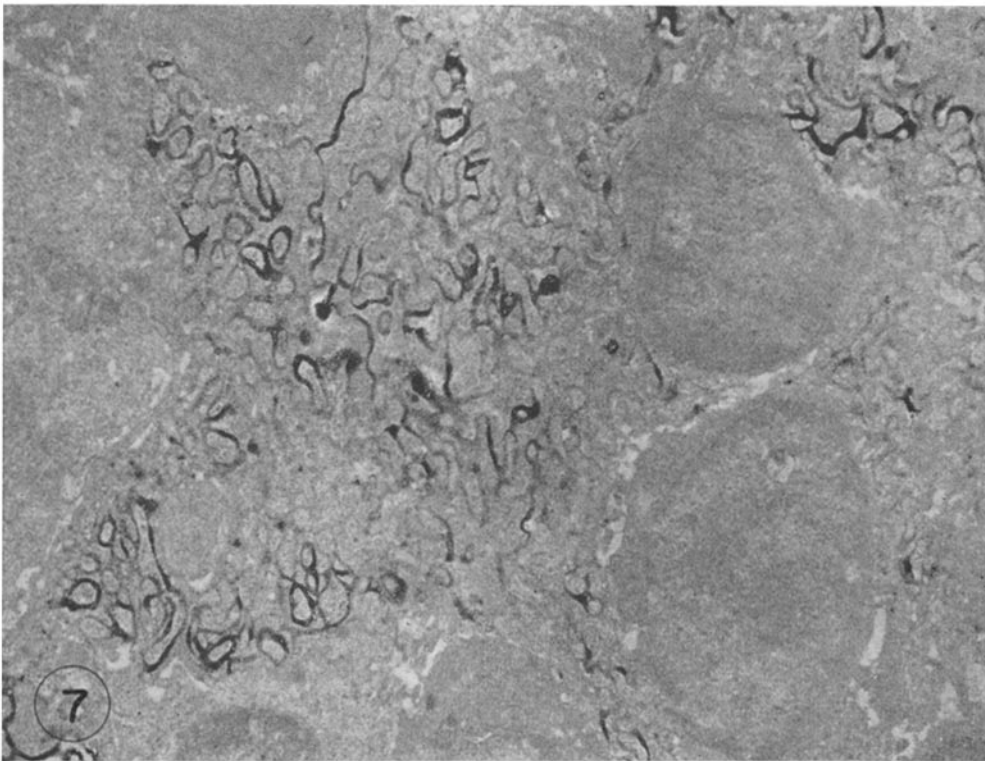
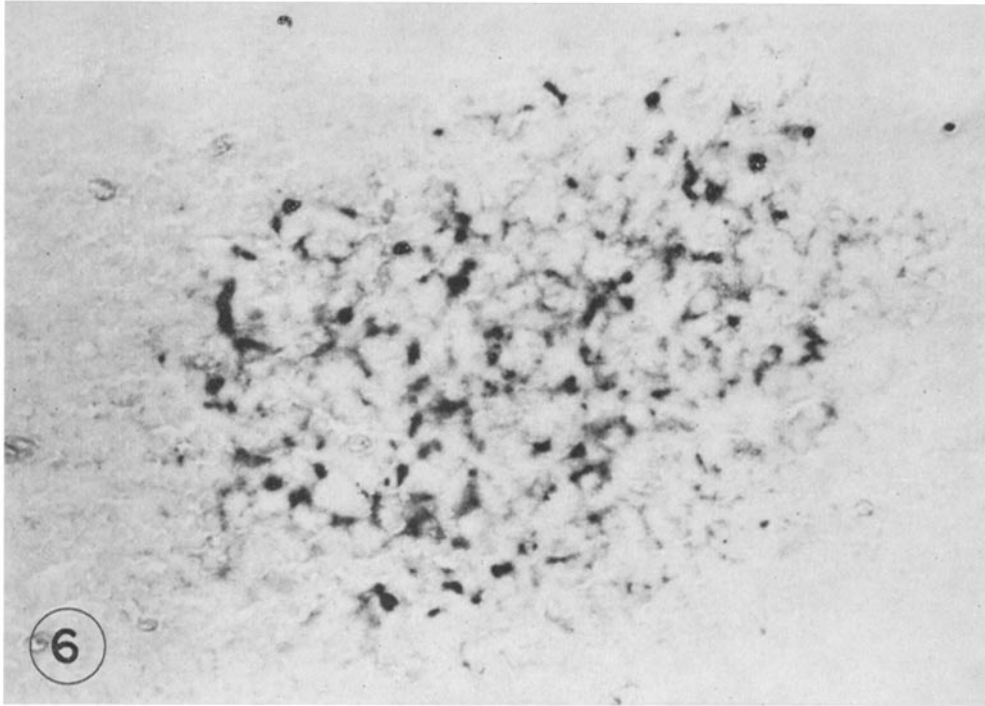


FIG. 6. Predominantly intercellular localization of anti-HRP antibody in a germinal center, 35 days after primary stimulation. $\times 250$.

FIG. 7. Electron microscopic aspect of a germinal center similar to that shown in Fig. 6, 35 days after primary stimulation. Extracellular localization of anti-HRP antibody which appears to be concentrated between dendritic reticular cells. $\times 5800$.

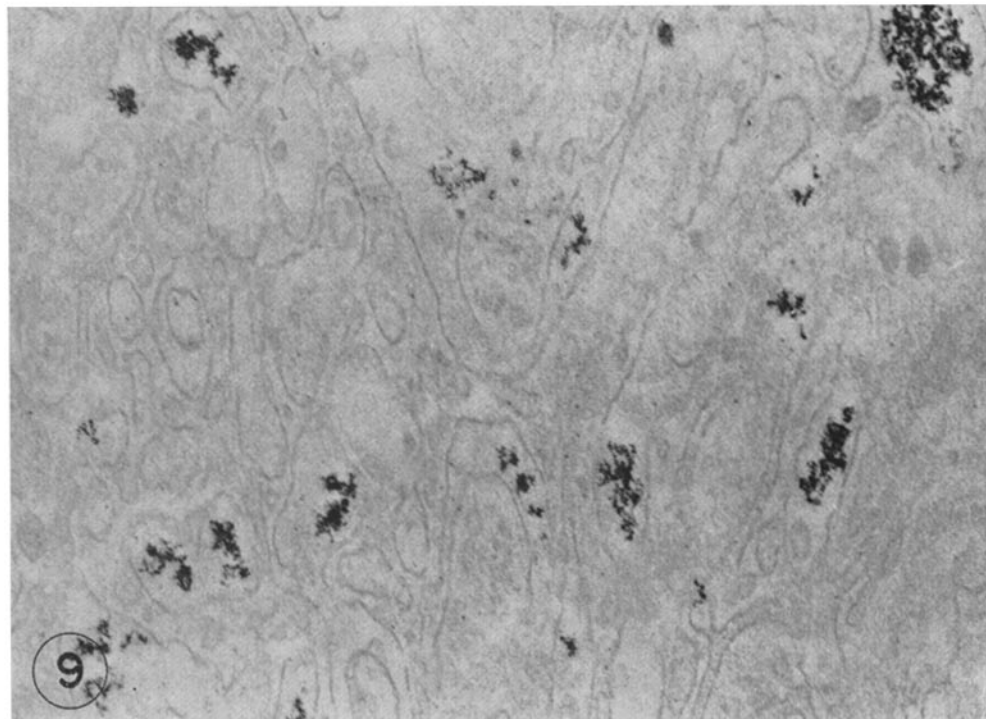
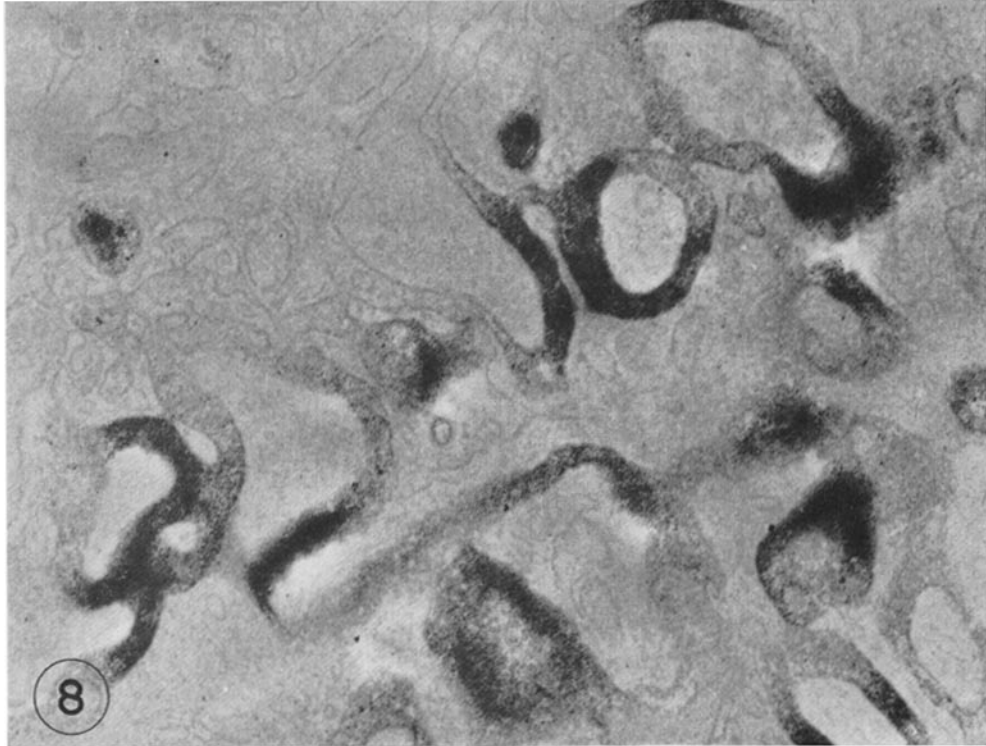


FIG. 8. Enlargement of an area comparable to that seen in Fig. 7. Varying amounts of the antibody-enzyme reaction product between dendritic processes of reticular cells, 35 days after primary stimulation. $\times 50,000$.

FIG. 9. Fine granular deposits of antigen (HRP) in the space between dendritic processes of reticular cells, 35 days after primary stimulation. $\times 64,000$.